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DOI: https://doi.org/10.1159/000329667

Kinane DF, Mombelli A (eds): Periodontal Disease. Front Oral Biol. Basel, Karger, 2012, vol 15, pp 1–16

# Subgingival Biofilm Structure

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**Abstract**

Periodontitis is an inflammatory disease of the oral cavity initiated by a microbial biofilm (or 'dental plaque'). Subgingival biofilms in periodontal pockets are not easily analyzed without the loss of structural integrity. These subgingival plaques are structured communities of microorganisms with great phylogenetic diversity embedded in a self-produced extracellular polymeric matrix. For almost three decades, knowledge of the structure of plaque located below the gingival margin has been limited to landmark studies from the 1970s that were unaware of the breadth of microbial diversity we appreciate now. Only recently has technical progress – combining histology, confocal scanning fluorescent microscopy and fluorescent in situ hybridization to localize the most abundant species from different phyla and species associated with periodontitis – provided new insights into the architecture of subgingival biofilms. This review focuses on the structure and composition of subgingival biofilms and discusses current knowledge on the nature of the extracellular matrix. We describe further structural aspects of 'subgingival' biofilms produced in vitro that are gaining considerable interest as we search for models to investigate biofilm development, resistance to antibiotics, extracellular polymeric matrix composition and function, and reciprocal host-cell-to-biofilm interactions.



Microbial dental plaque, regardless of differences in location and composition, consists of adherent consortia of microorganisms (mostly bacteria, few archaea, viruses, yeasts, amoebae, and a virtually unknown, presumably large, population of bacteriophages) that fulfill the consensus definition of biofilms. This definition describes '. . .a bacterial biofilm as a structured community of bacterial cells enclosed in a self-produced (hydrated) polymeric matrix and adherent to an inert or living surface' [1]. Subgingival biofilms are 3D structured communities of bacteria that live attached to the surface of the root of teeth or dental implants, with their outer surface directly facing the gingival tissue. In a healthy periodontium, these sites are not accessible to bacteria. However, persistence of biofilm at the gingival margin and in the gingival sulcus leads to gingivitis, a reversible condition, which in susceptible patients may

progress to periodontitis characterized by the irreversible loss of the tooth-supporting structures.

A key feature of this destructive inflammatory process is the formation of deep gingival pockets, which are colonized by the biofilm as they develop. The biofilm bacteria are embedded in a poorly understood extracellular matrix composed of exopolysaccharides, proteins and extracellular DNA. The formation of subgingival biofilms and its continuous adaptation to changing environmental conditions is governed by a dynamic equilibrium between the microorganisms, the cellular and humoral host defense, and a multitude of anabolic and catabolic products and signaling factors produced by both the microbiota and the periodontal tissues [2]. This results in a complex biofilm ecology where bacteria behave fundamentally differently than in suspension cultures where they exhibit a planktonic existence.

Max A. Listgarten, a pioneer of structural analyses of oral biofilms, while still at UPenn in 1994, published an excellent review on 'the structure of dental plaque' [2], which summarized the relevant work done up to that time point and highlighted the concepts on the relationship of biofilm structure to clinical status and the clinical relevance of biofilm composition and structure. The review was written at a time when new molecular microbiological techniques were beginning to conquer the dental field [3]. Eventually, this new development led to an explosion of information on the complexity and diversity of biofilm composition not imaginable at that time. It is the intent of this chapter to build on the review of Listgarten [2] and update our current understanding of subgingival biofilms in light of the progress that the technical and conceptual developments of the last 15 years have brought with regard to biofilm diversity, in vivo and in vitro biofilm architecture, and extracellular matrix composition.

## Improved Analytical Procedures Reveal Complex Subgingival Biofilm Composition

Extensive culture analyses performed in the 1980s and early 1990s to determine the predominant cultivable subgingival microbiota showed that this biofilm may harbor as much as $10^9$ bacteria and more than 100 different species in a single pocket [4, 5]. If the microbiota of entire study cohorts rather than individual pockets are considered, much larger diversity is apparent and suggests that, on a population basis, more than 500 bacterial species might be found in samples from the human oral cavity [4]. Some subgingival species, such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Tannerella forsythia,* stood out in that they were found with increased prevalence and in clearly elevated numbers at diseased sites in comparison to healthy control sites. In 1996, these species were designated as periodontal pathogens in the Consensus Report of the World Workshop in Periodontology. Today they figure among the best-studied medically important bacteria, and in many respects have become model organisms. Nevertheless, it was clear all the time that other cultivable

and non-cultivable subgingival species may be equally important and would merit further investigation [5]. Spirochetes, accounting for as much as 50% of the microscopically detectable subgingival microbiota, may serve as a prime example.

Do we need to describe the microbial community structure in a taxonomically precise and quantitative way? Amann and Ludwig [6] raised this question in a review 10 years ago and answered it with: 'Yes, of course!' Why? Because complex microbial ecologies cannot be characterized sufficiently by selected individual taxa that may have adapted particularly well to their ecological niche. Complex microbial ecologies are rather defined by all the mutual interactions that determine the abundance, localization and activities of their members [6]. Clearly, new tools for studies of (oral) microbial diversity were required. These became available in the mid-1980s with the emergence of new procedures for the comparative sequencing of homologous biopolymers [7]. In particular the extensive sequencing of ribosomal RNA (16S and to a lesser extent 23S rRNA) became very attractive and influential as mirrored by the now more than $1.9 \times 10^6$ deposited 16S rRNA sequences (Ribosomal Database Project August 2011; http://rdp.cme.msu.edu). In 2001, Paster et al. [8] described 215 novel phylotypes associated with subgingival plaque. In the following years, numerous conceptually and technically similar studies complemented each other and lengthened the list of newly recognized species and phylotypes, culminating in the creation and most recently in an update of the Human Oral Microbiome database (www.homd.org) [9]. This database includes a backbone library of about 800 16S rRNA gene sequences that are structured phylogenetically and taxonomically into 619 taxa in 13 phyla. The backbone library will be expanded in the near future by data from an additional 36,000 clonal sequences of which approximately 10% – accounting for as much as 654 new taxa – are not yet covered by the current Human Oral Microbiome backbone database [9]. In addition to this, recent progress in DNA sequencing technology ('pyrosequencing') has brought an enormous increase in nucleotide sequences of oral microbial origin, resulting in an overwhelming number of phylotypes of unknown species affiliation [10–12]. Where in the biofilms are the taxa located? How abundant are they? What are their morphologies, their physiological activities, their importance for both biofilm and host-biofilm relationship? To a large part we do not know – yet. Recently, technical progress has provided very promising possibilities for the single-cell identification and localization in intact natural subgingival biofilms of most taxa and phylogenetic clusters with an available 16S rRNA sequence.

## Methods to Study Subgingival Biofilm Architecture

Some of the most valuable information on supragingival biofilm formation, ecology and architecture has come from in situ studies performed with volunteers wearing expoxy crowns [13], artificial surfaces attached to selected teeth [14], or specifically designed appliances into which enamel, dentin, glass or plastic slabs had been inserted

[e.g. 15–17]. While all these studies could only identify the biofilm bacteria based on morphology or cell wall structure (if electron microscopy was used) and differentiate between live and dead organisms using vitality stains, some more recent studies used fluorescent in situ hybridization (FISH) technologies to identify targeted biofilm bacteria at the species level [e.g. 18–21]. The application of phylogenetic group- or species-specific single cell identification techniques to undisturbed biofilms formed supragingivally in the oral cavity on retrievable surfaces is currently the state of the art, and promises to reveal important new information on supragingival plaque formation and architecture.

Unfortunately, the study of subgingival plaque is much more difficult in comparison to supragingival plaque due the protected location of periodontal pockets. Accordingly, the development of the 3D subgingival biofilm structure is less well characterized. Access to undisturbed natural subgingival biofilms can only be gained by tooth extraction. Listgarten and co-workers [22] pioneered structural analyses of oral biofilms more than three decades ago using light and electron microscopy. The major hindrance of their groundbreaking studies was the impossibility at the time to identify the detected bacteria beyond cell morphology (cell shape, cell wall structure and Gram stain). This obstacle has only recently been overcome when entire teeth affected by advanced periodontitis were extracted without disturbance of the adherent subgingival biofilm and – after immediate fixation and processing to serial sections of 2 μm thickness – were stained by FISH using various combinations of group- and species-specific rRNA probes and studied by epifluorescence or confocal laser scanning microscopy (CLSM) [23]. A way to investigate in situ subgingival biofilm formation without the loss of the tooth under study was developed by Wecke et al. [24], and over the last few years has been refined and applied in several studies [25–27]. This procedure uses gold foil or small plastic carriers covered with expanded polytetrafluoroethylene membranes, which are inserted to the depth of the periodontal pockets for defined periods of time and then processed similarly to extracted teeth. In the following, these recently published studies are reviewed in greater detail as they may lead the way to further investigations that likely will be carried out using much broader selections of phylogenetic group-, genus- and species-specific FISH probes to elucidate the subgingival biofilm architecture.

## Subgingival Plaque Structure

A profound understanding of the in vivo structure of subgingival biofilms, i.e. the natural situation, is essential for designing and interpreting diversity measurements and in vitro experimental biofilms. Histological slices perpendicular to the root surface reveal three easily envisioned and clinical relevant areas, namely the bottom of the pocket, the root surface and the epithelial side [28]. At the bottom of the pocket, the periodontal ligament and the gingival connective tissue border the biofilm. This is

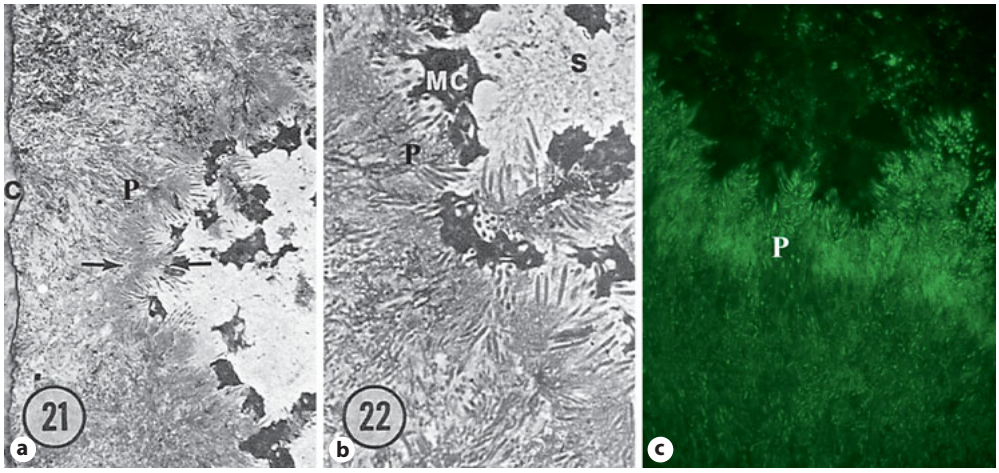


**Fig. 1.** Overview of subgingival biofilms taken in the 1970s and 2008 (**c**) from extracted teeth. **a**, **b** Electron microscopic views. Reproduced with permission of the American Academy of Periodontology [21, 22]. **c** Stained with a FITC-labeled universal bacterial probe that stains the bacteria green. Note the remarkable agreement in biofilm structure and observed cell morphologies. C = Cementum surface; MC = mammalian cells; P = palisading bacteria; S = spirochete-rich region.

the area where further attachment loss occurs in progressing periodontitis and where further pristine tooth surface becomes colonized. The species or bacterial cell morphologies observed in this area are filamentous, large rod-shaped, spirochete-shaped and branching. Especially the branching cells are abundant, whereas spirochetes are sometimes missing [29].

The bulk of the biofilm mass is situated along the root surface with histologically defined parts adhering to the root surface and facing the gingival host tissue. Listgarten [22] was the first to describe the structure of such subgingival biofilms in 1976 using light and electron microscopy. His pioneering work with natural teeth affected by periodontal disease provided the first informative glimpses at subgingival plaque structure. The pictures of subgingival biofilms showed a wealth of different cell morphologies like cocci, rods, fusiforms, spirochetes, flagellated bacteria, small and very large forms, and bacterial aggregates. Moreover, the biofilm itself showed a distinct organization, indicating that biofilms might be structured entities themselves that are beneficial to their inhabitants.

The images of figure 1 show a remarkable and unprecedented agreement between samples taken 30 years apart and analyzed with different techniques [22, 23]. They provide a clear overview of the different structures that can be distinguished when analyzing subgingival biofilms. Three different zones are observed between the root surface and the gingival tissue lining of the periodontal pocket. In the zone directly adhering to the root surface, relatively small cells without any particular orientation are visible (fig. 1, 2). These bacteria are embedded in somewhat fibrous membrane-

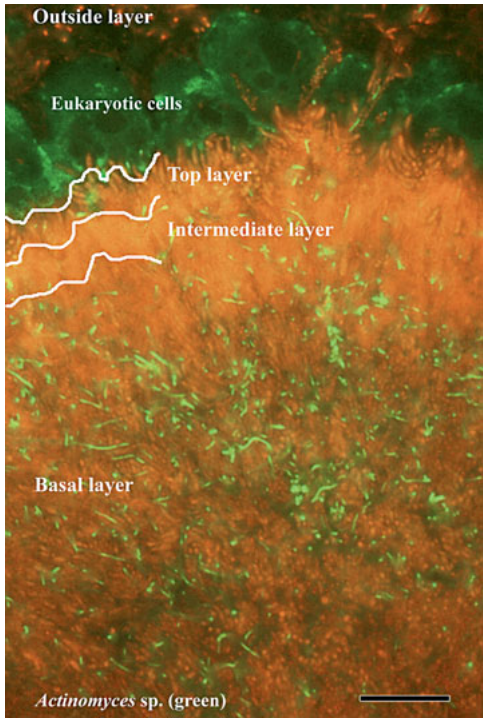


**Fig. 2.** Overview of the subgingival biofilm with *Actinomyces* sp. (green), bacteria (red) and eukaryotic cells (large green cells on top). The four different layers of the biofilm are depicted with the root surface orientated to the bottom of the image [for technical details, see 23]. Scale bar = 10 µm.

like material that may consist of debris, dead cells and biofilm matrix components. Amongst these unidentified bacteria with various morphologies, other bacteria can be recognized as *Actinobacteria* due to their characteristic branching or labeling by FISH (fig. 2) [23]. On top of that layer, a structurally different layer of bacteria can be observed that is more compact and electron dense (fig. 1, 2). This typical intermediate layer contains distinctive bacteria that are filamentous or rod shaped, and is without a well-defined intercellular matrix [22, 30]. FISH has identified these bacteria in part as *Fusobacterium nucleatum* and *T. forsythia* (fig. 3) [23]. The outermost part of the adhering biofilm that is facing the host tissues is characterized by a palisading layer of so-called 'test tube brushes' and of large parallel cells that are orientated with their long axis perpendicular to the root surface (fig. 1, 2). The test tube brushes consist of one or several large central filaments surrounded by gram-negative rods or small filaments. The parallel-orientated cells have been identified as members of the *Synergistetes* phylum (fig. 4) [23, 31], and represent a lineage composed of taxa that for the most part are not yet cultivable. Opposing the *Synergistetes* in this outermost part of the adhering biofilm, polymorphonuclear neutrophils can be observed by light, electron and epifluorescence microscopy (fig. 1, 2, 4, 5) [22, 28]. Several reports described an additional loose layer of bacteria covering the adhering biofilm without a clear cellular organization and dominated

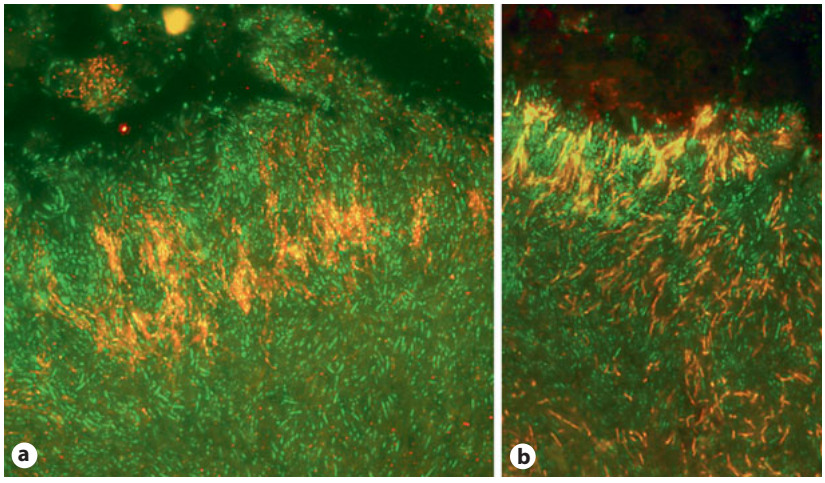


**Fig. 3.** The intermediate layer of the biofilm is dominated by fusobacteria (yellow, **a**) and *T. forsythia* (yellow, **b**). Modified from Zijnge et al. [23]. Scale bars = 10 µm.

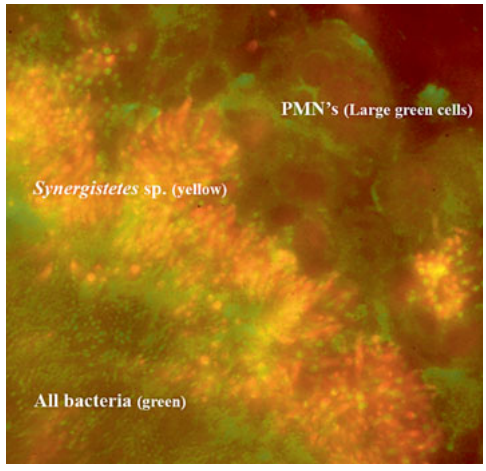


**Fig. 4.** Detailed overview of the top layer of the biofilm where *Synergistetes* sp. (yellow), visible as large perpendicularly orientated cells, are opposing large eukaryotic cells (green), most likely polymorphonuclear leukocytes. Modified from Zijnge et al. [23]. Scale bars = 10 µm.

by flagellated and spirochetal cells belonging to *Selenomonas* and *Treponema* species [23, 26, 32], although rods, filaments, cocci and test tube brushes could also be observed (fig. 6). A clear demarcation between the host tissue and this loose bacterial layer is not recognizable, probably because severe inflammation induces immunopathological tissue damage accompanied by collagen loss, ulceration and marked tissue permeabilization.

The subgingival biofilm shows a stratification from the root surface towards the epithelium lining. The basal, intermediate, top and outer layers seem to provide a

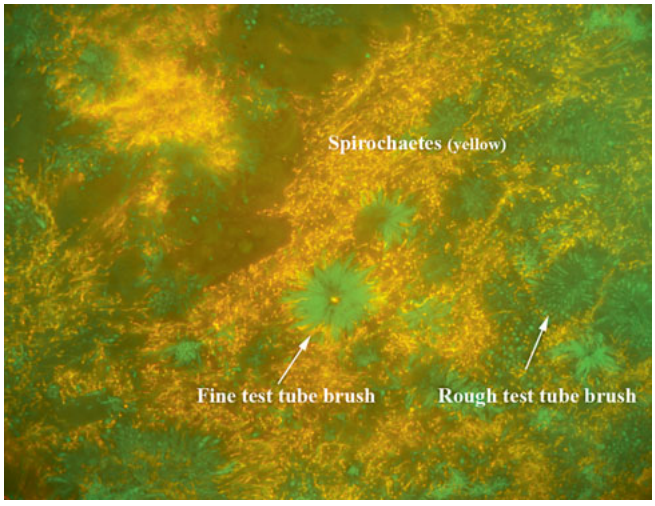


**Fig. 5.** Detail of the outside layer showing the dominance of *Treponema* sp. (yellow) and the presence of two types of test tube brush (arrows). Modified from Zijnge et al. [23].

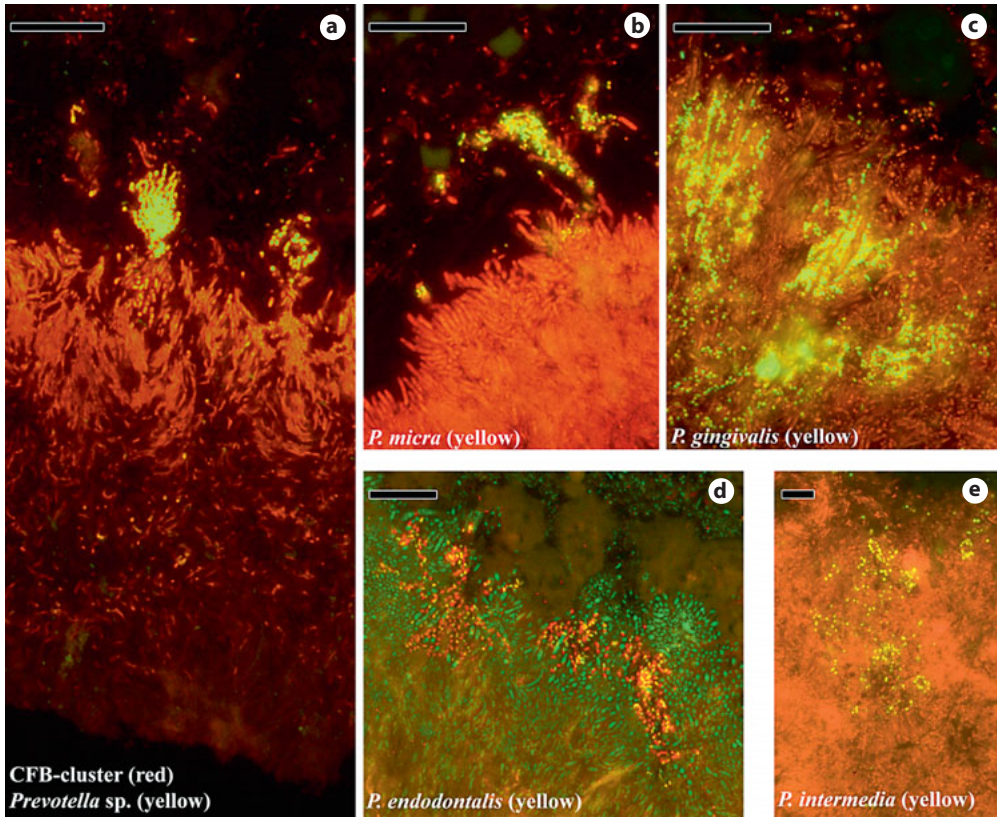


**Fig. 6.** Localization of typical periodontitis associated species in the biofilm. **a** Overview of the subgingival biofilm with *Cytophaga-Flavobacterium-Bacteroides* (CFB)-cluster species (red) and *Prevotella* sp. (yellow). Since *Prevotella* sp. are part of the CFB-cluster of bacteria, cells appear in yellow. **b** Top of the biofilm with a microcolony of *P. micra* (yellow). **c** Microcolonies of *P. gingivalis* (yellow) in the top layer. **d** Microcolonies of *P. endodontalis* (yellow) in the top layer. **e** Microcolonies of *P. intermedia* in the top layer. In panels **b–e**, bacteria are universally stained with a red or green label. Reproduced from Zijnge et al. [23].

general structure of the biofilm with corresponding species associated with each layer. *Actinomyces* sp., *F. nucleatum*, *T. forsythia*, *Synergistetes* sp. and *Treponema* sp. appear to make up a framework of the subgingival biofilm. Observations with species-specific probes and gold-labeled antibodies further show species that colonize within this biofilm framework by forming distinct microcolonies. In the outer layer of the adhering biofilm, closest to the epithelium and cells of the host defense, aggregates or microcolonies of *Prevotella intermedia*, *Porphyromonas endodontalis*, *P. gingivalis* and *Parvimonas micra* (formerly *Peptostreptococcus micros*) have been observed (fig. 5).

Microbial biofilms are dynamic communities exposed to an ever-changing environment. Therefore, the outlined architecture of the subgingival biofilm should not be seen as a rigid structure, but rather as a continuously changing consortium that is influenced by bacterial growth, attachment and detachment, the host's inflammatory immune response, the host's oral hygiene measures, and the nutritional conditions defined by the ecological niche. The availability or lack of nutrients is of course directly linked to both biofilm composition and extent of inflammation. Although the general setup of the subgingival biofilm framework appears relatively uniform, less common species may be observed in diverse sites, in particular within the loose layer that covers the adhering biofilm.

Clearly, our understanding of the subgingival plaque structure is incomplete. The number of teeth studied, the number of patients who provided teeth, and the number and specificities of the employed FISH probes are too limited for more precise conclusions. The degree of diversity currently appreciated among plaque organisms [9] is not reflected in the currently available studies of biofilm architecture. The application of rRNA FISH probes with specificities for large phylogenetic groups – such as β-, γ- or ε-Proteobacteria, *Bacteroidetes*, *Eubacteria* sp., *Selenomonas* sp. – to subgingival plaque samples that had been dispersed and subjected to FISH on multi-well glass slides, indicates that other bacterial taxa may account for substantial amounts of the biofilm [Gmür et al., unpubl. data].

## Structure of 'Subgingival' Biofilms Formed in vitro

It is evident that these natural subgingival biofilms are extremely complex and sited in a barely accessible, fluctuating and inflammation-affected environment. Together with ethical restrictions, this limits in vivo experimentation and makes interpretation of results difficult. Several research groups have developed in vitro model systems of subgingival plaque with the aim of reducing complexity while maintaining the bacterial biofilm 'lifestyle' and reproducing characteristic properties of such communities [e.g. 33–36]. Regardless whether freshly collected dispersed human subgingival plaque or balanced mixtures of strains of subgingival species were used to initiate biofilm formation, the nutritional conditions were recognized to have a dominant influence on

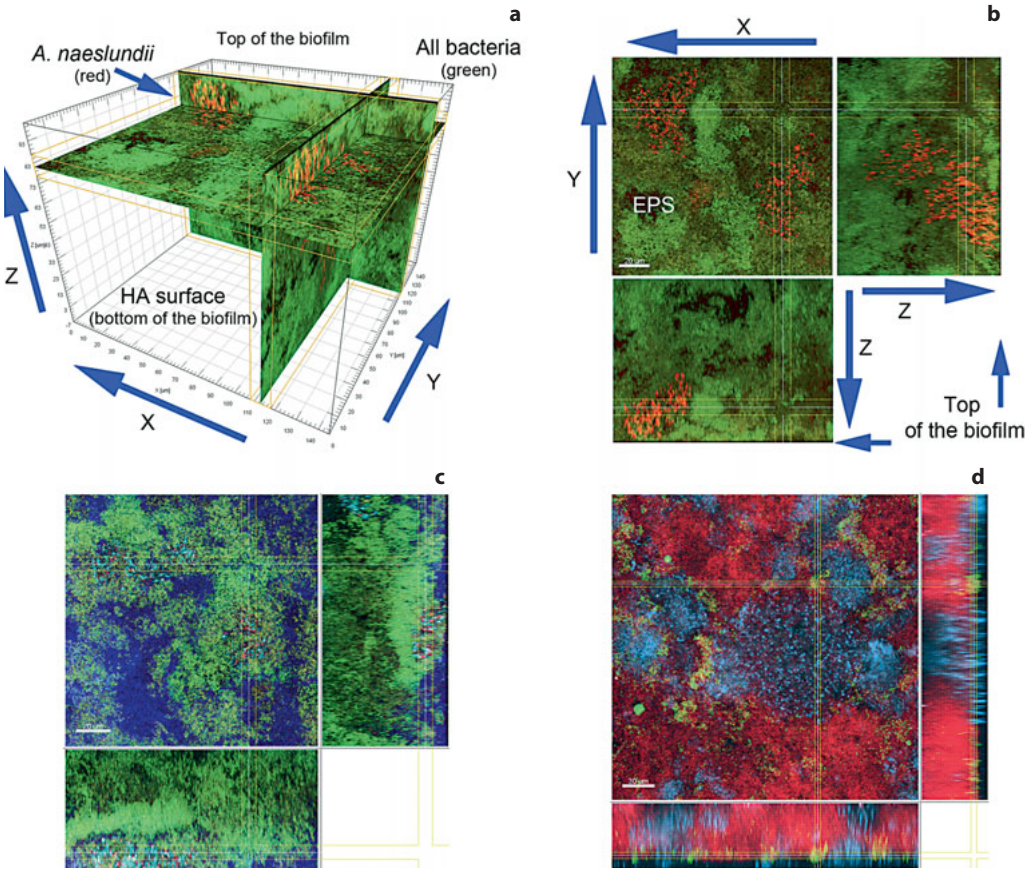


**Fig. 7.** CLSM images of 10-species 'subgingival' in vitro biofilms grown in parallel for 64.5 h on hydroxyapatite (HA) discs in 50% serum/50% medium and then fluorescence-labeled by multiplex FISH. **a** 3D arrangement of transverse (X-Y), sagittal (Y-Z) and coronal (X-Z) CLSM sections shown in the other panels. **a**, **b** Sections through the same biofilm stained for *A. naeslundii* (red, probe L-Act476–2-Cy3) and all bacteria (green, SYTOX green/YO-PRO). Black areas are bacteria-free spaces filled by extracellular polymeric substances (EPS). Scale bar = 20 µm. **c** Biofilm stained for *T. denticola* (light blue, TrepG1_679-Cy5), *P. gingivalis* (red, L-Pgin1006–2-C3), all bacteria (green, Sytox and YO-PRO) and extracellular polysaccharides (dark blue, Calcofluor). Note that the two specifically labeled species are located in close proximity to each other near the surface of the biofilm. Scale bar = 20 µm. **d** Spatial distribution of *S. anginosus and S. oralis* (both blue, STR405-Cy5) and *F. nucleatum* (red, Fnuc133c-Cy3) demonstrating their abundance and formation of large self-aggregates. The green-yellow stained bacteria are *V. dispar* (VEI217-FAM), characteristically located in the neighborhood of streptococci and at the top of the biofilm. Scale bar = 30 µm. Images are from Ammann et al. [unpubl. data].

biofilm development [35, 36]. This must have profound effects on biofilm structure, but information on the architecture of such in-vitro-generated subgingival biofilms is still scarce [34, 36]. Figure 7 shows images from our ongoing studies [Ammann et al., unpubl. data] investigating the architecture of 10-species 'subgingival' biofilms grown

in vitro in a saliva-free standard fluid medium supplemented with human serum (50:50%). The biofilms reached a thickness of 70–100 μm after 64.5 h of incubation with all inoculated species at detectable levels. *F. nucleatum, Streptococcus oralis* and *Streptococcus anginosus* predominated and were found throughout the biofilm (fig. 7d). Streptococci did not grow dispersed but formed large compact aggregates, often surrounded by layers of *Veillonella dispar* (fig. 7d). *A. naeslundii* was detected in relatively large individual colonies (fig. 7a, b) embedded in a readily detectable extracellular polysaccharide matrix (not shown) and spreading from the bottom third to the top of the biofilm. This suggests that *A. naeslundii* expanded rapidly from a relatively limited number of foci and, with a little longer incubation period, would have possibly spread over the entire body of the biofilm. This would correspond to the spatial distribution seen with in vivo biofilms (fig. 2). In contrast, *Treponema denticola* and *P. gingivalis* were detected selectively in close proximity to each other atop of very dense brightly stained cushions of bacteria (presumably streptococci) at the biofilm surface (fig. 7C). The presence of spirochetes and *Porphyromonas* species at this location is a characteristic attribute of subgingival plaque (fig. 5, 6); hence, it will be important to further analyze in vitro the reproducibility of this interesting finding. It must be emphasized that these are preliminary data gained with a limited number of biofilms. However, they demonstrate that the combination of 3D CLSM and multiplex FISH promises to be of great value in further elucidating the architecture of both natural and artificially generated subgingival biofilms.

## Extracellular Matrix Composition

There are as many different types of biofilms as there are bacteria [37]. Nevertheless, in any biofilm a substantial part consists of material other than bacteria. In general it is estimated that microorganisms account for less than 10% of the dry weight of biofilms and that the extracellular matrix contributes over 90% [38]. The formation of the extracellular matrix is generally recognized as the second stage during biofilm formation, after initial adherence and proliferation of microorganisms [39]. The extracellular matrix is composed of a heterogeneous mixture of polysaccharides, proteins and extracellular DNA (eDNA), which are called collectively extracellular polymeric substances (EPS). These complex conglomerate EPS are very difficult to analyze. Hence, it is not surprising that current knowledge of biofilm-associated EPS is derived almost exclusively from single-species biofilm models. However, whether the EPS production by these bacteria is the same when they live within a complex 'biofilm-city' [40] remains to be seen.

Extracellular polysaccharides occur in two basic forms. They can be associated with the cell surface and form a capsule or be secreted as a slimy biofilm matrix. Most polysaccharides are long linear or branched molecules composed of multiple saccharide units like glucose, fructose or sucrose and exist as homo- or heteropolysaccharides.

Many of them possess acyl-groups and organic and inorganic substituents like acetate, pyruvate or sulphate. These substituents and the presence of charged sugar residues largely determine the physical properties of the extracellular polysaccharides. The type of polysaccharide that is produced usually varies among the different species and some species can produce multiple kinds of polysaccharides [37]. One of the most commonly studied matrix polysaccharides is β-1,6-*N*-acetyl-D-glucosamine called PNAG or PGA. It is produced, among others, by *A. actinomycetemcomitans* for biofilm formation. In *A. actinomycetemcomitans* PGA mediates intercellular adhesion and contributes to biofilm cohesion, but also protects the cells against killing by macrophages [41]. *Prevotella nigrescens* produces a heteropolysaccharide that is composed mainly of mannose, but also contains other sugars, including glucose and fructose [42]. A mannose-rich polysaccharide has also been identified from *P. intermedia* [43, 44]. The polysaccharides produced by *P. nigrescens* and *P. intermedia* contribute to biofilm formation and the resistance to neutrophil phagocytosis. *P. gingivalis* strains on the other hand produce a capsular polysaccharide. Encapsulated strains have been shown to evade the immune system [45] but non-encapsulated strains are more adherent to epithelial cells and show strong autoaggregation and enhanced biofilm formation [46, 47]. If and what type of free extracellular polysaccharide *P. gingivalis* produces for the formation of biofilms is unknown. Actinobacteria produce levan which is a homopolysaccharide composed of β(2r6)-linked fructosyl units [48]. Levan may function as extracellular storage polymers, but has also been shown to stimulate the inflammatory response. The contribution of levans to actinobacteria biofilm formation is unknown.

Extracellular proteins that can be found in, and contribute to, a biofilm are lectins and sugar-binding proteins that facilitate cell-to-cell or cell-to-matrix interactions [49]. A second group of extracellular proteins involved in cell-to cell or cell-to-matrix interactions are autotransporters. Autotransporters transport themselves across the outer membrane of gram-negative bacteria and may function in adherence and biofilm formation. A third group of extracellular proteins that structurally contribute to the biofilm matrix are pili, also called fimbriae [37]. A type I and a type IV pilus are distinguished. The type I pilus consists of a long rigid structure of repeating subunits that is attached to the cell with a fimbrial tip that recognizes specific substrates. Type I pili have been observed on a number of oral bacteria [50] including *P. gingivalis*, *A. naeslundii* [51] and *P. intermedia* [52]. In *Actinomyces oris*, type I pili are functional in biofilm formation [53]. Type IV pili are often located at one of the poles of the cell and may provide the cell with twitching motility. The pilus is a fiber composed of repeating units of pilin encoded by the *PilA* gene [54]. Type IV pili have been identified in *Eikenella corrodens* and *A. actinomycetemcomitans* [50, 55]. Genomic screening indicated the presence of type IV pili as in *F. nucleatum* [54]. In *A. actinomycetemcomitans*, the type IV pilus is considered to be a distinct subtype assembled as bundles of long thin fibrils encoded by the *tad* locus [55]. *A. actinomycetemcomitans* strains deficient in the formation of pili form relatively fragile biofilms [56].

Extracellular DNA has been shown to be an important constituent of biofilms and to contribute to biofilm integrity [38, 57, 58]. There are indications that eDNA results from the controlled lysis of cells as well as from the active release of DNA containing membrane vesicles by viable cells [37, 38]. It is tempting to speculate that eDNA might function as a grid along which bacteria can move through the biofilm by using type IV pili with DNA-specific binding sites. There is no doubt that the characteristics of the EPS contribute to the specific properties of the biofilm. Many components are hygroscopic and keep the biofilm hydrated and prevent the cells from drying. The EPS matrix can act as a molecular sieve and protect the bacteria in the biofilm from toxic compounds or host immune responses, and nutrients are accumulated. The enhanced resistance of biofilms in comparison to planktonic bacteria to antimicrobial agents is well established [59]. The entanglement of biopolymers provides the biofilm with the physical strength and flexibility to withstand mechanical forces and shear stress. While biofilms provide an optimal environment for bacterial growth and survival, external impacts that may cause starvation or altered bacterial crosstalk are known to induce the production of enzymes that can potentially degrade EPS components. For example, *A. actinomycetemcomitans* produces the extracellular *N*-acetyl-β-hexoseaminidase dispersin B that hydrolyzes the glycosidic links in PNAG and degrades the biofilm matrix. This may result in the dispersal of cells or clumps of cells, which in turn can colonize new surfaces and initiate a new biofilm lifecycle [38, 57]. Flemming and Wingender [38] concluded in their recent review that 'despite much research on biofilms, basic questions remain' and continued that 'a better understanding of the regulation of EPS production in mixed-species biofilms, as well as a spatial and temporal dissection of the phases in EPS production, will reveal important aspects of the oldest, most successful and widespread form of life on Earth'.

## Acknowledgement

T.A. is supported by the research fund of the Swiss Dental Association.

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